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Generation of Ribozymes by Rolling Circle Transcription of Promoterless Single-Stranded DNA Circles in Mammalian Cells

[Ribozimlerin, Promotorsuz Tek-iplikli DNA Çemberlerinin *Rolling Circle* Transkripsiyonu Ile Memeli Hücrelerinde Üretimi]

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ABSTRACT

Self-processing hairpin ribozymes have been synthesized from promoterless single-stranded DNA circles (73 nt) within mammalian cells. Following lipid-mediated transient transfection, DNA circles were efficiently internalized by mouse L cells (OST7–1) that stably express T7 RNA polymerase confining it to the cytoplasm. Cellular uptake of circular DNA templates and intracellular accumulation of ribozyme RNA transcripts from these DNA circles were progressive, both peaking at 24 h after transfection. Intracellular transcription generated RNA concatemers accumulating to a level of ~100 copies per cell. Transcription appears to be independent of specific promoter sequences but depends on T7 RNA polymerase. The data presented here may support the hypothesis that single stranded bubble regions within duplex DNA can serve as *de novo* initiation sites for RNA transcription not only *in vitro* but also in the cytoplasm of mammalian cells. These results may provide a model for the rolling circle transcription of small circular nucleic acids in mammalian cells.

Key Words: T7 RNA polymerase, RNA polymerase II, *rTth* DNA polymerase, rolling circle transcription, multimeric RNAs, ribozyme catalysis, circular DNA

ÖZET

Tek iplikli, promotorsuz çembersel DNA'lardan (73 nt) kendisini kataliz edebilen firkete ribozimler memeli hücrelerinde sentezlenmiştir. Lipitlere dayalı transfeksiyon yöntemi ile DNA çemberleri, sürekli T7 RNA polimeraz üreten ve bunu sitoplazmada tutan fare L hücreleri (OST7–1) tarafından başarılı bir şekilde içeri alınmıştır. Kalıp DNA çemberlerinin hücrelere alınması ve hücre içi ribozim transkriptlerinin sentezi zamana bağlı olmuş, her ikisi de 24 saatte maksimum düzeylerine ulaşmışlardır. Hücre içi transkripsiyon sonucu konkatemerik RNAlar sentezlenmiş ve bunların seviyesi her hücrede ~100 kopyaya ulaşmıstır. Transkripsiyon bilinen bir promotor dizisinden bağımsız ancak T7 RNA polimerazına bağımlı olarak gelişmiştir. Burada verilen bilgiler, DNA dupleksindeki tek iplikli balon bölgelerinin RNA transkripsiyonu için yalnız *in vitro* değil aynı zamanda memeli hücre sitoplazmalarında da *de novo* başlangıç bölgelerinin olabileceği hipotezini desteklemektedir. Bu sonuçlar, küçük nükleik asit çemberlerinin memeli hücrelerinde "*rolling circle*" transkripsiyonu için bir model oluşturabilir.

Anahtar Kelimeler: T7 RNA polimeraz, *rTth* DNA polimeraz, "rolling circle" transkripsiyon, multimerik RNA'lar, ribozyme katalizi, çembersel DNA

INTRODUCTION

Recent studies have demonstrated that RNA synthesis can in some cases be initiated at specific structures rather than at specific promoter sequences. Single stranded "bubble" regions within duplex DNA may serve as *de novo* initiation sites perhaps mimicking the open transcription complex formed by the RNA polymerase [1–9]. Small single-stranded circular DNA oligonucleotides encoding catalytic RNA molecules can serve as templates for initiation and elongation of RNA sequences including self-processing hammerhead, hairpin, and hepatitis delta virus (HDV) ribozymes in the absence of primers, promoter sequences, and duplex DNA structures [5–7,9]. Preliminary results suggest that transcription can be more efficient on a circular DNA template than on a linear one, since RNA polymerase can transcribe the same sequence multiple times without dissociation [5–7,9]. Quite small circles (16–34 mer) can be used as substrates by some common polymerases such as Klenow fragment, and T7 and *E. coli* RNA polymerases [7,10–12]. This rolling circle transcription (RCT) can be employed to synthesize concatemeric and self processed unit length catalytic RNA sequences without any appended sequences. The RCT reaction under *in vitro* conditions results in high levels of amplification [6,7] by T7 and *E. coli* RNA polymerases. Recently the RCT synthesis has been shown to take place in *E. coli* [13]. Although *in vitro* RCT reactions on a variety of DNA circles are successful, the initiation of the transcription and its sequence dependence are not well understood. It has been proposed that some secondary structure preferences may play a role in transcription initiation on these templates [7]. RNA concatemers generated by transcription of circular DNAs encoding self-cleaving hairpin, hammerhead, and HDV ribozymes are self-processing and generate unit length linear monomers, multimers and circular RNAs. Analogous RNA transcripts generated from plasmid DNA vectors have been shown to undergo self-processing within mouse L cells (OST7–1) that express T7 RNA polymerase, and confine it to the cytoplasm [14,15]. Furthermore, recent preliminary studies have indicated that DNA nanocircles could also be transcribed in OST7–1 cells [16].

In the present study we have further investigated the possibility of generating *trans*-acting ribozymes from DNA nanocircles within the mammalian cells. To ensure that the molecules studied *in vitro* and in cells are as comparable as possible, synthetic promoterless DNA circles (73 nt) encoding a self-processing hairpin ribozyme were delivered into OST 7–1 cells. Cellular internalization of the DNA and expression of ribozyme transcripts were monitored and characterized. Our results demonstrate the detection of RNA products expressed from promoterless single-stranded DNA circles in the cytoplasm of OST 7–1 cells and this reaction depends on cellularly expressed T7 RNA polymerase. These results suggest that RNA polymerase remains bound to the template generating RNA concatemers via a rolling circle transcription mechanism. The data presented here offer several possibilities for intracellular generation of viroid and virusoid-like RNA molecules with catalytic activity within mammalian cells from DNA nanocircles encoding nothing but the desired transcript.

MATERIALS AND METHODS

Synthesis of linear and circular DNA templates

The sequences and synthesis of DNA circles were described previously [7].

Transcription and *in vitro* RNA processing

In vitro transcription was performed as described [7,14]. Briefly, 1 μM of circular and linear DNA templates were transcribed with either T7 RNA polymerase or RNA pol II. A PCR amplicon (0.5 μM) encoding a self-cleaving hairpin ribozyme from a T7 promoter sequence was used as a double-stranded DNA control [14]. Reactions (10μl) contained 40 mM Tris•HCl (pH 8.0), 25 mM $MgCl_2$ (10 mM $MgCl_2$ and 1mM $MnCl_2$ for reaction with RNA pol II) 5 mM DTT, 1 mM spermidine, 0.5 mM each ATP, UTP, GTP, CTP, 12.5 units pancreatic RNase inhibitor (Ambion, Inc.), 0.25 μl (10 mCi/ml, [^{32}P] CTP, 3000 Ci/mmol), 1 μl T7 RNA polymerase (20 units/μl), 1μl RNA pol II. Reactions were incubated at 37°C for 3 h then treated with 2 units of DNase I (Ambion, Inc.) for 15 min at 37°C. The products were mixed with equal volume of formamide loading buffer containing 25 mM EDTA, denatured at 90°C for 2 min and resolved on a 10% (w/v) polyacrylamide (19:1 acrylamide:bis-acrylamide) /8 M urea gel with a 1xTBE (Tris-borate/EDTA, pH 8.72) buffer. Gels were run either at room temperature or at 4°C at 30 W. The gels were analyzed using a Bio-Rad GS–525 molecular imager and Molecular Analyst 2.1 software.

Ribozyme activity assays

In vitro single turnover cleavage kinetics were performed using gel purified linear monomer, dimer and trimer RNA species. Substrate sequence was 5'-CUGUAGUCCAGGAA–3' as described (HIV–1 pol 3605–3618, strain HXB2; 8,13). Substrate was mixed with standard cleavage buffer (50 mM Tris•HCl pH 8.0, 12 mM $MgCl_2$). Reactions were initiated by adding ribozyme into the premixed substrate and cleavage buffer. Single-turnover cleavage kinetics with isolated ribozyme species (300 nM ribozyme, ~1 nM of 5'-labeled substrate) were performed either in the standard cleavage buffer or in 40 mM Tris•HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermine and 25 units of RNase inhibitor [7] Ambion) at 37°C. Reactions were resolved on a denaturing gel and results were quantified as described [14]. The fraction of substrate cleaved was plotted versus time and fit to the single exponential equation by nonlinear re-

gression analysis using SigmaPlot 4.14 software (Jandel Scientific) as described [14].

Topology analysis of RNA transcripts

RNA processing (cleavage/ligation) reactions were examined using internally labeled RNA transcripts. DNase I treated and purified RNA transcripts were incubated in 50 mM Tris•HCl (pH 7.5) and 1 mM Co $(NH_3)_6Cl_3$ at 25°C [17]. Aliquots were removed at time intervals (0 min to 20 h) and quenched with an equal volume of formamide loading buffer containing 25 mM EDTA, denatured at 90°C for 2 min and resolved by a 10% denaturing sequencing gel electrophoresis. Gels were run either at room temperature or at 4°C at 60 W. The linear and circular 73 nt DNA templates used in the transcription reaction were resolved by a 10% denaturing gel electrophoresis but stained with ethidium bromide in order to determine the relative mobility of the DNA templates relative to linear and putative circular RNA species.

In addition, the above RNA transcript was treated with snake venom phosphodiesterase I (SV1, USB). Exonuclease time course cleavage of RNA transcripts were examined by incubating the reaction in 50 mM Tris•HCl (pH 8.0) and 12 mM $MgCl_2$ and 0.00025 units SV1 at 37°C [18]. Aliquots were removed at time intervals (0 min to 20 h) and samples were processed as described above.

RNA sequencing

Sequences of gel purified and 5'-end-labeled linear monomer, dimer, and trimer RNA species were determined essentially as described [7,18,19] by partial hydrolysis with RNase T1, U2, and alkaline hydrolysis (50 mM Na_3PO_4, pH 12, at 50°C for 5 –15 min).

In vitro RNA transcripts were used for sequencing reaction by primer extension as described [14]. The primer used for sequencing was 5' -GGAGGTACCAGGTAATGTACC- 3'. RNA transcript (*ca*.3 pmol) was first denatured in the presence of 5'–^{32}P-labeled DNA primers (4×10^5 dpm, *ca*. 40 fmol) at 80°C for 10 min in 5 µl hybridization buffer (60 mM NaCl, 50 mM Tris•HCl (pH 7.5), 5 mM DTT) and then slowly cooled to 30°C to permit annealing of primers to their complementary RNAs. Annealed samples (2 µl) were then added to 3 µl of sequencing buffer (0.375 mM dNTPs, 0.1 mM of the appropriate dideoxy ribonucleoside triphosphate in 1x hybridization buffer). Reverse transcription reactions were initiated by adding $MgCl_2$ to 8 mM, and 5 units of AMV reverse transcriptase (USB) followed by a 30 min incubation at 47°C. Reactions were quenched and resolved on a denaturing sequencing gel.

Transfection of OST7–1 cells and extraction of total cellular RNA and DNA

OST7–1 cells grown in 60 mm plates to a confluency of ca. 60–70% [14,15] and were transiently transfected using lipofectamine-plus or lipofectin reagent (Invitrogen) as described [14]. To monitor the time course of DNA uptake and intracellular accumulation of RNA transcripts, 2 µg of DNA were used to transfect the cells as described above except that the cell lysates were collected at 0, 12, 24, 36, 48 hours post-transfection. Total cellular RNA was extracted as described [14], and total cellular DNA was also extracted following manufacturers instructions (Trizol, Invitrogen).

DNA and RNA dot-blot analysis

Cellular total DNA (1 µg) or RNA (5 µg), and aliquots of culture media (10 µl) obtained from the transfected cells were blotted on a nylon membrane (Nytran Plus, 0.2 µm, S&S) using the Bio-Dot microfiltration apparatus (Bio-Rad) in accordance with the manufacturer's instructions. Membranes were prehybridized with (150 µl/cm^2) of 1 mM EDTA, 7% SDS, 0.5 M Na_2HPO_4 (pH 7.2) for 5 min at 65°C. For hybridization, prehybridization solution was replaced with the same solution. A 5'-labeled DNA probe (1×10^6 dpm/ml) was denatured for 5 min at 100°C and added into the hybridization reaction and incubated at 43°C for DNA blot and 44°C for RNA blot for 24 h. The probe used for DNA blot is complementary to the circular DNA precursor (5'-CTCCCTGTAGTCCAGTTTTCG–3') and the probe used for RNA blot is same as the one used for primer extension. Post-hybridization washes were performed twice in (350 µl/cm^2) 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), 5% SDS and in 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2), 1% SDS at 53°C for DNA blot and at 43°C for the RNA blot for 1h for each wash solution. Membranes were exposed for autoradiography without further drying.

Asymmetric PCR

Sensitive asymmetric PCR (linear amplification by primer extension) reactions were performed on total DNA isolated from the transfected cells to monitor the fate of the DNA circles. A 5'-labeled primer (1×10^6 dpm *ca*. 0.006 µM) complementary to the circular DNA template (see above) was included in reaction solution consisting of 50 mM KCl, 10 mM Tris•HCl (pH 8.3),1 mM $MgCl_2$, 1.25 units of Taq DNA polymerase (Perkin Elmer-Cetus), 0.4 mM ddGTP, 0.2 mM dATP, dCTP and dTTP, respectively to generate discrete chain termination products (it yields a ddGTP-terminated 29 nt product) and *ca.* 100 ng of total cellular DNA and 1 µl of cell culture media containing transfected cells. The reaction was subjected to 22 cycles of PCR (94°C, 45 sec; 95°C, 15 sec; 47°C, 1 min; 72°C, 1 min and final extension of 72°C 60 min). The products were separated on an 8% denaturing sequencing gel.

Cycle-poisoned-primer extension

This sensitive assay is a modification of reverse transcriptase primer extension assay using chain terminated nucleotides [20,21]. The 5'-labeled primer (4×10^5 dpm

ca. 0.01 µM) was annealed to 1 µg total RNA and extended with 0.8 units of r*Tth* DNA polymerase (Perkin Elmer Cetus) in a solution containing 1 mM $MnCl_2$, 90 mM KCl, 10 mM Tris•HCl (pH 8.3), 0.4 mM ddTTP, 0.2 mM dATP, dCTP, dGTP, and *ca*. 3 µCi of [^{32}P] dATP (10 mCi/ml, 3000 Ci/mmol), respectively to generate discrete termination products. The primer (5' -GGAGGTACCAGGTAATGTACC- 3') is complementary to the 3'-end of the hairpin ribozyme, and yields a 28 nt termination product from both unprocessed and processed RNA transcripts. The reaction was subjected to 45 cycles (85°C 45 sec, 88°C 15 sec, 50°C 1 min, 70°C 2 min and final extension of 70°C 30 min). After 25th cycle a fresh aliquot of *rTth* DNA polymerase (0.8 units) was added into each reaction. The products were resolved on a 14% denaturing sequencing gel.

RESULTS

In vitro transcription and RNA processing

Transcription reactions were carried out either with circular and linear DNA templates encoding a hairpin ribozyme (73 nt, 1 µM, Fig. 1) or with a double-stranded PCR amplicon (206 bp, 0.5 µM) encoding a self-cleaving hairpin ribozyme from a T7 promoter [14]. *In vitro* transcription of the DNA circles is efficient with T7 RNA polymerase relative to the transcription of the control DNA (Fig. 2). Transcription of the DNA circles generated long multimeric RNA segments suggesting that transcription proceeded through a rolling circle mechanism (Fig. 2). During transcription, RNA species were processed through a ribozyme-catalyzed mechanism generating unit length linear multimers and monomers (Fig. 2). In contrast, transcription of the linear template produced heterogeneous transcription products most of which were the size of unit length monomer and smaller. This is consistent with nonspecific initiation of transcription. No significant quantities of specific transcription products were observed for any of the templates when transcription reactions were carried out with RNA pol II isolated from eukaryotic cell nuclei (Fig. 2). Some low levels of full-length transcripts were detected from the templates used in the reaction. Linear control tem-

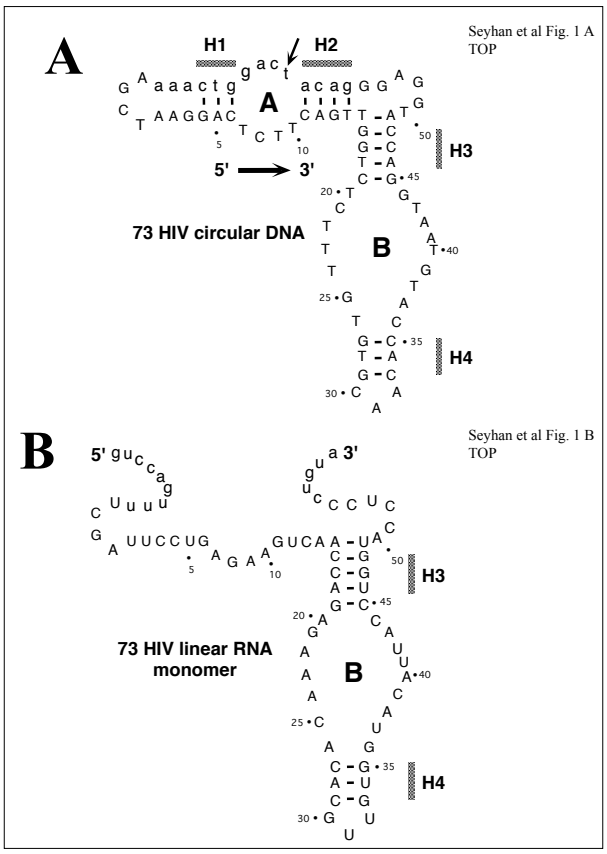

Figure 1. Schematic depiction and sequences of circular 73nt ssDNA, (A) and of self-processed hairpin ribozyme monomer (B). Substrate binding strand of the hairpin ribozyme is designed to target to HIV–1 pol (3605–3618) sequence. The encoded hairpin ribozyme is indicated by uppercase letters and substrate by lowercase letters. Arrow indicates encoded cleavage site. Heavy arrow indicates the 5'- >3' strand orientation.

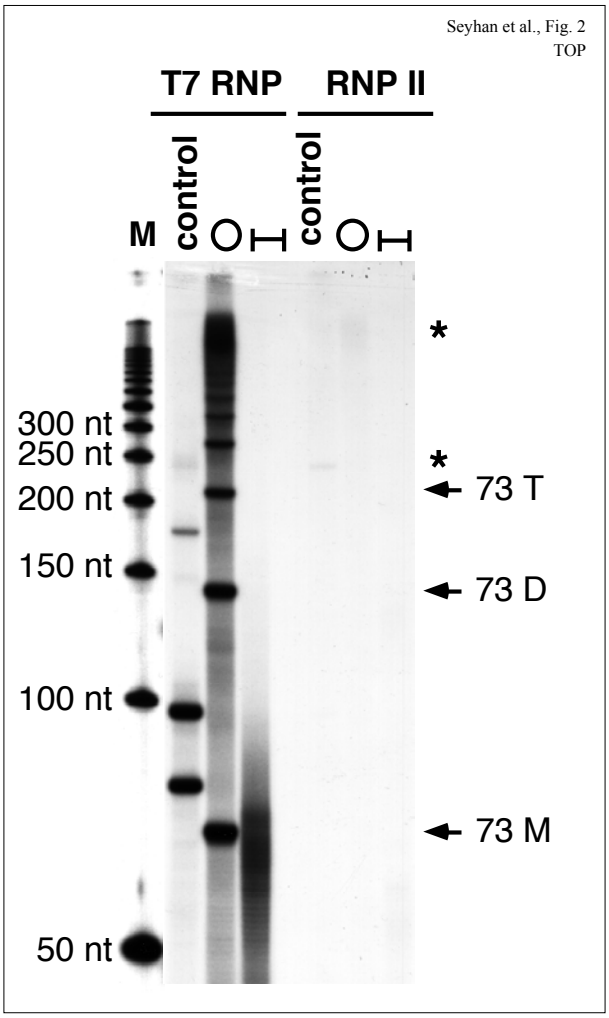

Figure 2. Schematic depiction and sequences of circular 73nt ssDNA, (A) and of self-processed hairpin ribozyme monomer (B). Substrate binding strand of the hairpin ribozyme is designed to target to HIV–1 pol (3605–3618) sequence. The encoded hairpin ribozyme is indicated by uppercase letters and substrate by lowercase letters. Arrow indicates encoded cleavage site. Heavy arrow indicates the 5'- >3' strand orientation.

plate DNA and DNA circles generated only small quantities of RNA transcription products, and the amounts of RNAs produced were not sufficient to detect products of RNA processing. Transcription of the control template by RNA pol II generated products that were similar in size to the template DNA (206 nt) indicating that the initiation of transcription is nonspecific. Transcription of the circular and the linear templates by RNA pol II produced RNA transcripts similar in size to the transcription products generated by T7 RNA polymerase.

Self-processing reactions of RNA transcripts from single-stranded DNA circles

Self-processing reactions performed on RNA transcripts resulted in progressive accumulation of small quantities of circular monomeric RNA species when reactions were performed under conditions favoring product ligation while linear RNA multimers self-cleaved themselves, eventually yielding predominantly monomeric, dimeric, trimeric linear species (Fig. 2, 3B and 3C). During the self-processing reactions only a small amounts of monomeric circular RNAs were generated, apparently through a hairpin ribozyme catalyzed ligation reaction from the linear monomeric RNAs (Fig. 3B and 3C). Investigation of the relative mobility of these RNAs indicated that the linear and circular monomeric RNAs had the identical mobility with the DNA analogs on a denaturing polyacrylamide gel (Fig. 3C). In these experiments the mobility of the circular RNA differs from those reported previously, due to the gel electrophoresis conditions [7]. The stability of circular ribozyme species relative to linear species was tested with snake venom phosphodiesterase I, an exonuclease with 3' to 5' specificity. A time course cleavage reaction performed on internally labeled RNA transcripts showed a rapid degradation of linear RNA species while circular RNA species showed slower degradation over the course of 20 h (not shown), although circular ribozymes self-process themselves under these conditions generating linear monomers.

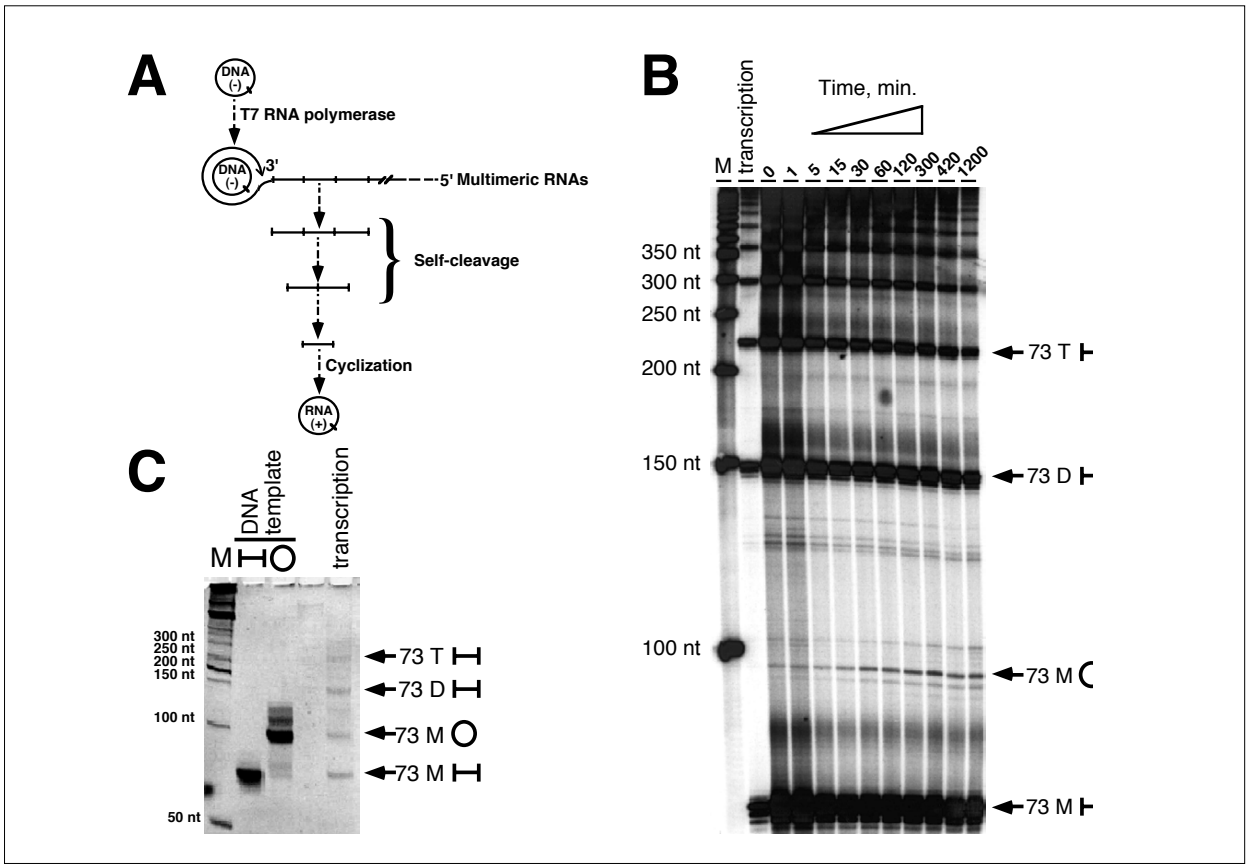

Figure 3. A: Schematic depiction of rolling circle transcription of DNA circles. Transcription yields long multimeric RNAs followed by self-processing and ligation through a hairpin ribozyme catalyzed mechanism mimicking the replication of naturally occurring viroids and virusoids. **B:** Time course of RNA processing reactions on RNA transcripts. Internally labeled in vitro RNA transcripts of the circular DNA template were incubated under self-processing conditions to generate the cyclized species. Purified RNA transcript was incubated in 50 mM Tris•HCl (pH 7.5) and 1 mM Co (NH_3) $6Cl_3$ at 25°C. Aliquots were removed at time intervals and quenched with an equal volume of formamide loading buffer containing 25 mM EDTA, denatured at 90°C for 2 min and the products were resolved on 10% denaturing gel. **C:** Topology analysis of DNA templates and RNA transcripts. Linear and circular 73 nt DNA templates used in the transcription reaction were resolved by 10% denaturing gel electrophoresis and stained with ethidium bromide to determine the relative mobility of the linear and circular DNA templates relative to RNA transcripts and putative circular monomeric RNAs. Product RNAs and template DNAs denoted by length and topology (73 M ı—ı, linear monomer; 73 D ı—ı, linear dimer; 73 M O, circular monomer; T ı—ı, linear trimer). M designates 50 bp double-stranded DNA size marker (Invitrogen).

Trans-cleaving activity of self-processing RNAs

Linear monomer, dimer and trimer RNA species were gel purified and characterized by standard ribonuclease sequencing methods as described in Materials and Methods (not shown) and examined for *trans*-cleavage activity. *Trans*-cleavage experiments using a 14 nt RNA substrate by these RNA species gave specific cleavage products (Fig. 4). The processed linear-monomer ribozyme cleaves substrate with a rate of 0.0056 min^{-1} (±0.0026) while the linear-dimer cleaved substrate at a slightly reduced rate, 0.0024 min^{-1} and the linear-trimer ribozyme cleaved substrate at a rate of 0.0018 min^{-1}. These rates should be considered maximum values, since during the *trans*-cleavage reaction, trimeric and dimeric ribozymes undergo further self-processing reactions generating linear dimers and eventually monomers. Inclusion of spermine in the reactions did not alter the results significantly. In all cases, the extents of cleavage exceeded 15% in 120 min. In the case of the circular RNA, previous studies showed that the circular monomer did not show *trans*-cleaving activity [7].

Cellular uptake of circular DNA templates

OST7–1 cells stably expressing T7 RNA polymerase were cotransfected with DNA nanocircles and a plasmid vector harboring a T7 RNA polymerase promoter-driven sequence encoding a self-processing hairpin ribozyme [14]. Primer extension analysis of the total cellular RNAs extracted from the co-transfected cells at time intervals gave signals specific to the plasmid DNA but not to the DNA circles. These data indicate that OST7–1 cells were being transfected and were expressing T7 RNA polymerase. However, accumulation of transcripts from the DNA circles was too low to be detected by a standard primer extension assay (not shown). Therefore, the uptake of circular DNA templates was monitored as a function of time. DNA dot-blot and asymmetric PCR analysis on total cellular DNA and on aliquots of cell culture media obtained at time intervals after transfection showed a progressive internalization of DNA templates (Fig. 5,6). DNA accumulation appeared to peak at 24 h then declined, apparently due to cell division and/or intracellular degradation of the template DNA. Analysis of cell culture media gave analogous results (Fig. 5) except that DNA templates were detectable through the course of transfection. Circular template DNAs appeared to remain relatively stable within the culture media throughout transfection. A noticeable difference of signal intensity was observed between DNA samples complexed with transfection reagent and naked DNA indicating that DNA-lipid complex is more stable in culture media. A series of titrated amounts of DNA circles was also used to assay the optimal DNA concentration for transfection. Little intracellular DNA was detected when 0.5 and 1 µg DNA were used for transfection (Fig. 5,6). However, significant amounts of

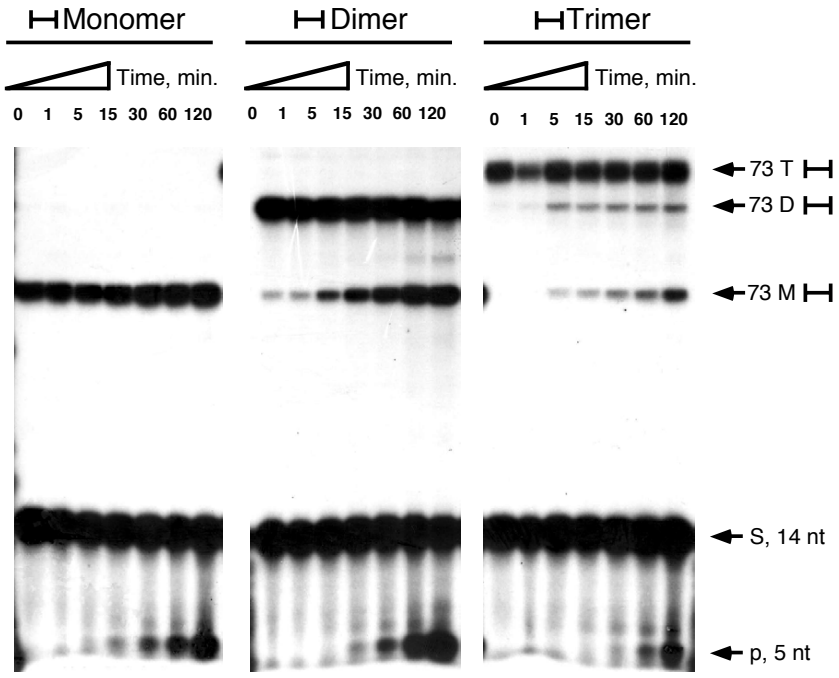

Figure 4. *In vitro trans*-cleavage of a synthetic 14 nt RNA substrate by isolated ribozyme species. *In vitro* single turnover cleavage kinetics were performed using internally labeled, gel purified linear monomer, dimer and trimer RNA species. Substrate sequence was 5'-CUGUAGUCCAGGAA–3' as described in Materials and Methods. Reactions were performed in the standard cleavage buffer as described [14] with ~300 nM of each ribozyme species and ~1 nM 5'-labeled substrate. Reactions were conducted at 37°C and both ribozymes and substrates were resolved by 20% denaturing gel electrophoresis.

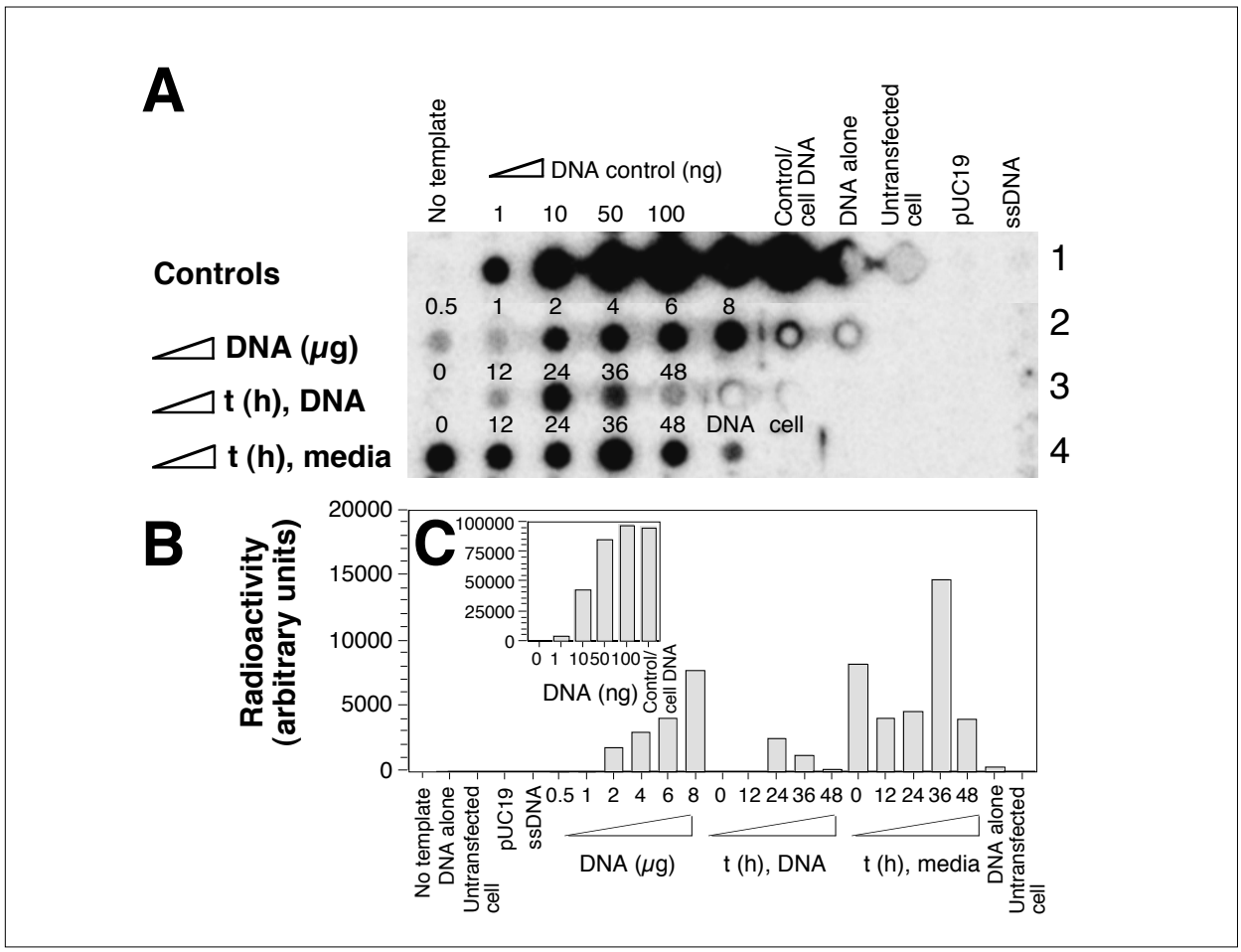

Figure 5. DNA dot-blot assay to monitor internalization of DNA templates by OST7–1 cells. **A:** Row 1: known quantities of synthetic DNA circles used for transfection were blotted directly onto the nylon membrane. Row 2: total cellular DNA (1 μg each) extracted at 48 h after transfection from the cells transfected with series of quantities of DNA templates were blotted. Row 3: total cellular DNAs (1 μg each) extracted at the indicated time points from the cells transfected with 2 μg of DNA templates were blotted. Row 4: aliquots of cell culture media (10 μl) obtained from the transfected cells at the indicated time points were blotted. Membranes were hybridized with a 5'-labeled DNA probe as described in Materials and Methods, exposed for autoradiography without further drying, and quantified using a Bio-Rad model GS–525 Molecular Imager and Molecular Analyst 2.1 software. No template, template DNA was omitted; control/cell DNA, 100 ng synthetic circular DNA and 1 μg of total cellular DNA; DNA alone and untransfected cell, either the transfection reagent or the template DNA was omitted in the transfection reactions. **B:** The plot represents the quantitation of the results. **C:** Inset represents the quantitation of the DNA dot-blot results, generated from known quantities of DNA circles (row 1).

intracellular DNA was detected when 2, 4, 6 and 8 μg of DNA used for transfection (Fig. 5,6). No detectable signal was observed from the DNA extracts of control transfections where either DNA or lipofectin was omitted or when heterologous DNAs (100 ng of each pUC19 and salmon sperm DNA) were used in the DNA dot-blot assay (Fig. 5, row 1). Results from the asymmetric PCR assay were in accordance with the DNA dot-blot results and the formation of specific PCR product confirms the specificity of the primer and the product (Fig. 6). The intracellular level of DNA circles was found to be ~87000 copies per cell estimated by comparing intensity of the 24 h time point signal obtained from the DNA extracts of cells transfected with 2 μg of DNA circles to known amounts of DNA of same species assayed by DNA dot-blot analysis (Fig. 5). Calculations were based on an estimated total cellular DNA content of 6 pg per cell.

Intracellular transcription from promoterless DNA nanocircles monitored by a sensitive primer extension assay

Total cellular RNAs isolated from transfected cells at time intervals were analyzed by RNA dot-blot (not shown) and by a sensitive primer extension assays (Fig. 7A). This assay is a modification of a reverse transcriptase primer extension assay using chain terminated nucleotides [20,21]. When the indicated dideoxynucleotides and deoxynucleotides are included, primer extension reactions are strongly terminated generating discrete termination products. We have further modified this assay by coupling it to an asymmetric amplification step (cycle-poisoned-primer extension). In this assay we used *rTth*, a thermostable DNA polymerase (Perkin-Elmer/Cetus) which can catalyze the polymerization of DNA using RNA as its only template in the presence of $MnCl_2$ [22]. Note that there is no DNA intermediate in

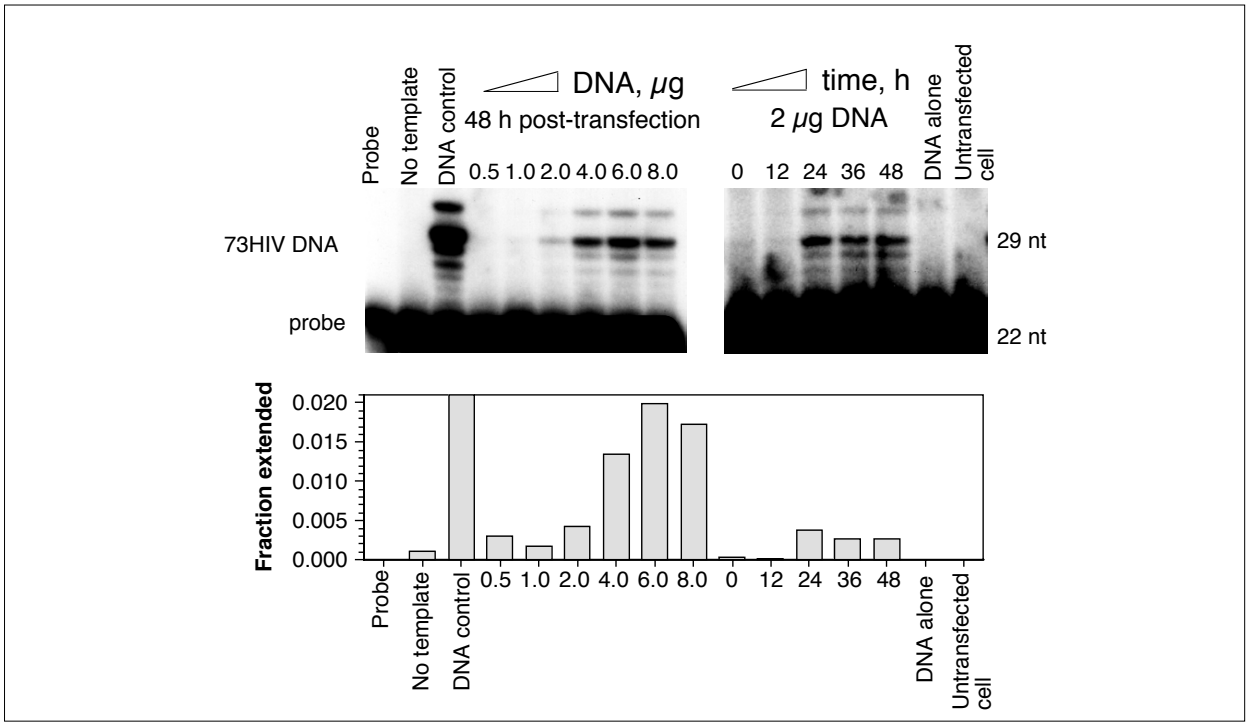

Figure 6. Asymmetric PCR to monitor internalization of DNA templates by OST7–1 cells. Top panel: asymmetric PCR reaction was performed on total DNA extracts isolated from the transfected cells to monitor the uptake and fate of the DNA circles as described in Materials and Methods. Probe, probe alone; no template, PCR reaction was performed without the template; DNA control, 100 ng circular DNA and 1 μg of cellular DNA; DNA alone and untransfected cell, either the transfection reagent or the template DNA was omitted in the transfection reactions. 73 HIV DNA represents the specific asymmetric PCR termination product generated from total cellular DNA extracted from the cells transfected with DNA circles. Bottom panel: the results were quantified as described and data was plotted as the fraction of extension products relative to sum of free primers and the extension products.

the reaction and only one primer specific to the RNA was used.

Analysis of total cellular RNAs isolated at the indicated time points from transfected cells with 2 μg of DNA circles showed progressive accumulation of ribozyme transcripts through the course of transfection within cells (Fig. 7B). Expression appeared to peak at 24 h after transfection which then declined, apparently due to cell division and/or intracellular degradation of the RNA transcript (Fig. 7B, C).

Intracellular steady-state levels of RNA transcripts was found to be less than 100 copies per cell estimated by comparing the intensity of the 24 h time point signal obtained from the RNA extracts to known amounts of *in vitro* RNA transcripts of same species assayed by RNA dot-blot analysis (not shown). Calculations were based on an estimated total cellular RNA content of 10 pg per cell [14,23,24]. In addition, the results demonstrated that the level of ribozyme transcripts were essentially identical at 48 h after transfection when cells were transfected with varied amounts of DNA circles (Fig. 7B, C) perhaps due to limiting amounts of cellular T7 RNA polymerase. Specificity of these products was confirmed by control reactions including a sequencing ladder performed on the *in vitro* transcribed same species of RNAs. No transcripts were detected in control transfections when either DNA or lipofectin was omitted or when control cells used that do not express T7 RNA polymerase (Fig. 7B, C and data not shown). Absence of signals at the early stages of transfection indicates that signals detected on RNA extracts are specific. The results obtained from both RNA dot-blot and cycle-poisoned-primer extension assay are in complete agreement both suggesting a progressive intracellular accumulation of ribozyme transcripts.

Results obtained from a cycled-primer extension assay carried out on the above RNA extracts where the chain termination step was omitted demonstrated the accumulation of long RNA transcripts consistent with intracellular synthesis of long RNA concatemers and/or generation of circular RNA transcipts that resulted in run around primer extension products. The product accumulation appeared to peak at 24 h after transfection but not at the early stages of transfection (not shown).

Discussion

Ribozyme RNAs are commonly generated by transcription of either a promoter-containing double stranded linear DNA template *in vitro* or circular plasmid-based vectors *in vivo*. Recent studies have shown that small single-stranded circular DNA oligonucleotides encoding catalytic RNA molecules could serve as templates for T7 and *E. coli* RNA polymerases for initiation and elon-

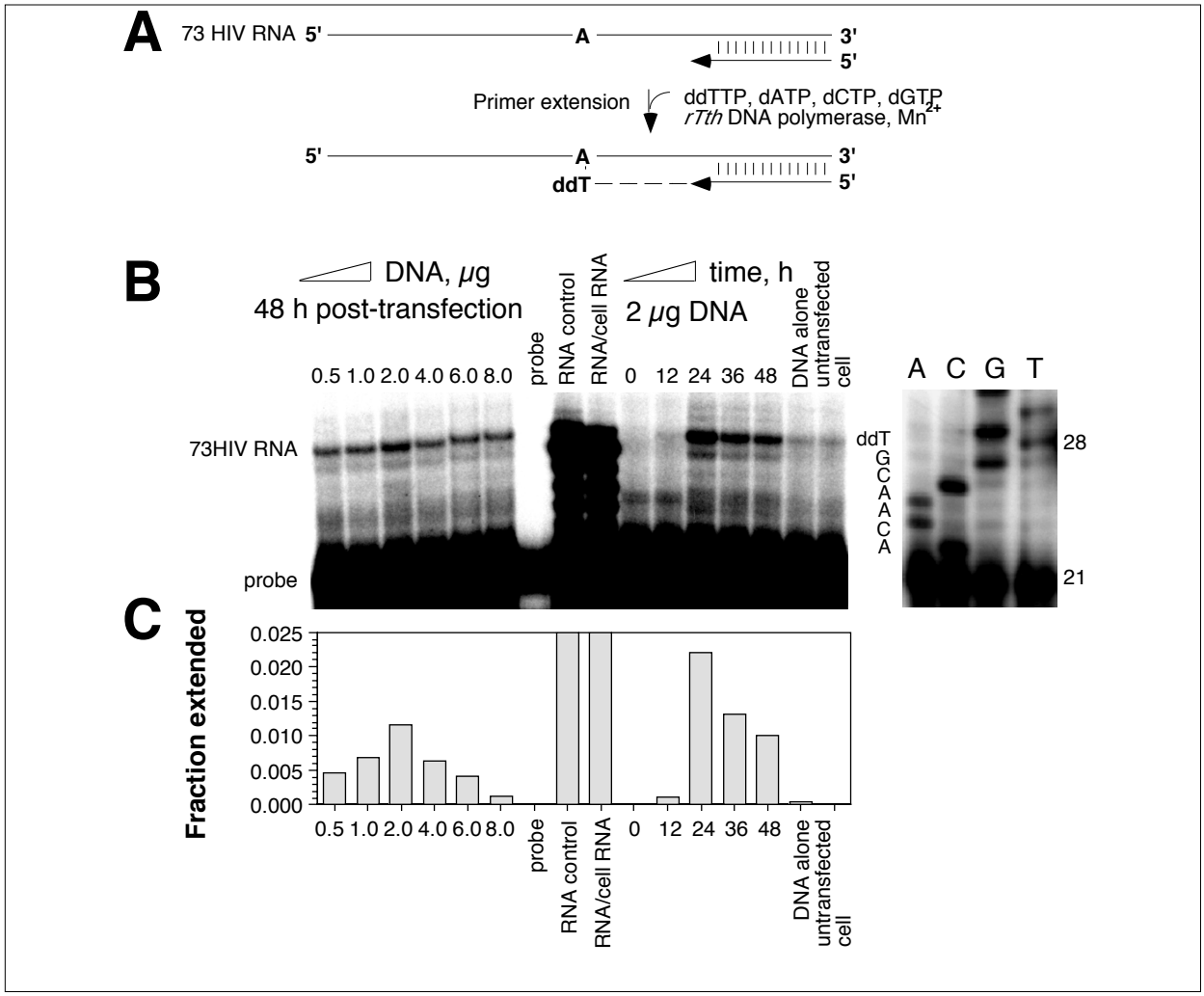

Figure 7. Intracellular accumulation of hairpin ribozyme transcripts expressed from DNA circles in the cytoplasm of OST7–1 cells. **A:** Schematic diagram of the sensitive primer extension assay to detect low quantities of RNA transcripts. When the indicated dideoxynucleotides and deoxynucleotides are included, primer extension reactions are strongly terminated generating discrete termination products. This reaction can be amplified on a thermal cycler when *rTth* DNA polymerase is used in the presence of manganese ion. **B:** A sensitive cycle-poisoned-primer extension assay was designed to monitor the intracellular expression of hairpin ribozyme transcripts from the DNA circles. Probe, probe alone; RNA control, RNA transcript (0.5 pmol); RNA/cell RNA *ca.* 0.5 pmol of RNA transcript was mixed with 1 μg of total cellular RNA; DNA alone and untransfected cell, either the transfection reagent or the template DNA was omitted in the transfection reactions. **C:** The bands were quantified as described and data was plotted as the fraction of extension products relative to sum of free primers and the extension products.

gation of RNA sequences [6,7,9]. Here we demonstrate the synthesis of self-processing hairpin ribozymes from promoterless synthetic single-stranded DNA circles (73 nt) in mammalian cells. Our results showed that internalization of DNA circles and intracellular accumulation of ribozyme RNA transcripts are progressive. T7 RNA polymerase is highly active *in vitro* and has shown modest activity in OST7–1 cells. RNA pol II showed only very little activity *in vitro*. Results obtained from a cycled-primer extension assay demonstrate the accumulation of long RNA transcripts, consistent with intracellular synthesis of long RNA concatemers and/or circular RNAs present in ribozyme containing RNA extracts. These results are also consistent with rolling circle transcription mechanism, the same mechanism thought to be used *in vitro* and similar to that viroid and virusoid RNAs [27,28].

RNA transcripts produced *in vitro* from the double-stranded DNA template (a PCR amplicon, 206 bp) and DNA from the nanocircles by RNA pol II were too little to detect any ribozyme processing products (only faint full length transcripts could be seen) indicating that this promoterless RNA synthesis is catalyzed efficiently by T7 RNA polymerase, but apparently not by RNA polymerase II. Transcription products were similar in size to the double stranded template DNA suggesting that the initiation of transcription is nonspecific. The high levels of *in vitro* activity of T7 RNA polymerase can be attributed to the fact that it is composed of a single polypeptide chain and thus does not employ a separate initiation factor. These same factors are important for the activity of the enzyme in the cytoplasm of OST7–1 cells. Intracellular accumulation of ribozyme RNA transcripts derived from the promoterless single-stranded DNA

circles were significantly lower than those obtained from the double-stranded DNA plasmids that contain T7 promoters. Several factors could account for the low levels of expression from the synthetic DNA circles: (i) suboptimum sequence and/or structure in the circle, (ii) inefficient cellular uptake, (iii) inappropriate subcellular localization, (iv) occlusion of DNA circles by cellular factors and lipofectin, (v) unstable RNA transcripts, and/or (vi) limiting intracellular concentrations of T7 RNA polymerase. No ribozyme specific signals could be detected when control cells that do not express T7 RNA polymerase were used. As for the first possibility, recent *in vitro* selection experiments from partially randomized DNA circles have shown that sequences undergoing more efficient transcription can be identified [13].

Although the intracellular levels of ribozyme containing transcripts were insufficient to permit the detection of ribozyme processing products, the observation that single-stranded DNA circles could be expressed in cells is potentially useful in the cellular application of ribozyme technology. In addition, these data support the hypothesis that single stranded bubble regions within duplex DNA can serve as *de novo* initiation sites for RNA transcription not only *in vitro* [7] but also in the cytoplasm of mammalian cells.

The results demonstrate that linear ribozyme species derived from self-processing reactions have RNA cleavage activity in *trans*, although these RNAs contain self-complementary sequences. This suggests that the self-complementary stems unfold to allow binding and cleavage of a substrate RNA in *trans*. *In vitro* activity of the isolated ribozyme species was lower than the standard *trans*-acting hairpin ribozymes [25], presumably because the additional sequences appended to the ribozyme RNA can compete effectively with the binding of the other RNA substrates in *trans,* thus may be acting as a rate limiting step subsequently reducing the catalytic efficiency of the ribozyme. It is possible that adjustment of the degree of self-complementarity will mitigate this interference; in the hammerhead ribozyme case the self-processed RNA ends having optimum self-complementarity do not slow the ribozyme activity and have the beneficial effect of greatly increasing sequence selectivity [26]. Finally, for the self-processed hairpin ribozymes, it was shown previously that the circular RNA species did not show RNA cleaving activity in *trans* [7], apparently due to the structural constraints of the circular RNA.

A small proportion of RNA transcripts produced by the RCT reaction is circular, and in some classes of ribozymes they are the chief products [9]. Circular RNAs are of interest partly because these RNAs are generated from a ribozyme- catalyzed mechanism in biologic systems [29,30] and because they are more stable against intracellular degradation [31]. However, methods for producing circular RNAs are limited to enzymatic treatment of linear RNAs [32,33] the group I intron splicing mechanism [34], *in vitro* hairpin ribozyme ligation mechanism [35], and template-directed cyclization of HDV RNA [9]. Our results suggest that self-processing mechanism by the hairpin ribozyme produces monomer length ribozyme sequences containing self-complementary ends, which can bind to the substrate binding sequence of the ribozyme by folding to form helices 1 and 2. This stabilizes the unit length ribozyme in a catalytically active conformation so that ligation can take place between the juxtaposing ends of the appended substrate sequences. The ligation products are not likely to be formed on the DNA template since the RNA transcripts were treated with DNase I which can attack both double or single stranded DNA [36]. Additionally, time course ligation experiments performed in the presence of the circular DNA template did not result in any additional circular RNA formation compared to the reactions where template DNAs were removed by DNase I treatment (not shown).

Our results demonstrate that a DNA nanocircles can be taken up and transcribed in the cytoplasm of mammalian cells, albeit at low levels, forming the predicted RNA products of rolling circle transcription. However, due to low level of intracellular expression, we were unable to detect self-processing products of multimeric ribozymes as monomers assuming that self-cleavage takes place in the cytoplasm of these cells [14]. The reasons for this could be two-fold: (i) the monomeric ribozymes are unstable and degraded rapidly by cellular nucleases, and/or (ii) the concentration of these RNAs were too little to detect as compared to the unprocessed multimeric products which were slightly more abundant and more stable. Nevertheless, these results are encouraging and may provide a basis for further development of intracellular expression of circular RNA transcripts and/or transcripts with self-binding domains as an effective way to stabilize small RNA molecules designed for therapeutic applications namely; ribozymes, small interfering RNAs (siRNAs), antisense oligonucleotides, RNA triplexes, or high affinity RNA ligands (aptamers or decoys). It could also provide a general method for expressing small RNA sequences that may be helpful in the study of medically important viroid-like human (HDV) and plant pathogens. However, further studies are needed to improve intracellular concentration of these RNA transcripts and to show that these ribozyme transcripts are capable of cleaving an RNA substrate in *trans*. Moreover, it will be necessary to identify sequence or structural motifs that allow transcription by cellular RNA polymerases and these studies are currently underway.

We also described in this study a modified chain terminated primer extension assay [20] by coupling it to a linear amplification by primer extension using *rTth* DNA polymerase and Mn^{2+}, where RNA is used as the sole template. We believe that the method described here is useful for directly detecting and quantitatively analysis of low copy RNA transcripts expressed in cells.

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